FLUORESCENCE ENERGY TRANSFER AS AN INDICATOR OF Ca^{2+}-ATPase INTERACTIONS IN SARCOPLASMIC RETICULUM

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ABSTRACT Ca^{2+}-ATPase molecules were labeled in intact sarcoplasmic reticulum (SR) vesicles, sequentially with a donor fluorophore, fluorescein-5'-isothiocyanate (FITC), and with an acceptor fluorophore, eosin-5'-isothiocyanate (EITC), each at a mole ratio of 0.25–0.5 mol/mol of ATPase. The resonance energy transfer was determined from the effect of acceptor on the intensity and lifetime of donor fluorescence. Due to structural similarities, the two dyes compete for the same site(s) on the Ca^{2+}-ATPase, and under optimal conditions each ATPase molecule is labeled either with donor or acceptor fluorophore, but not with both. There is only slight labeling of phospholipids and other proteins in SR, even at concentrations of FITC or EITC higher than those used in the reported experiments. Efficient energy transfer was observed from the convalently bound FITC to EITC that is assumed to reflect interaction between ATPase molecules. Protein denaturing agents (8 M urea and 4 M guanidine) or nonsolubilizing concentrations of detergents ($C_{12}E_8$ or lysolecithin) abolish the energy transfer. These results are consistent with earlier observations that a large portion of the Ca^{2+}-ATPase is present in oligomeric form in the native membrane. The technique is suitable for kinetic analysis of the effect of various treatments on the monomer–oligomer equilibrium of Ca^{2+}-ATPase. A drawback of the method is that the labeled ATPase, although it retains conformational responses, is enzymatically inactive.

INTRODUCTION

Interactions between Ca^{2+} transport ATPase molecules in sarcoplasmic reticulum (SR) may contribute to the mechanism of Ca^{2+} translocation and the regulation of the permeability of the membrane (Martonosi and Beeler, 1983; Martonosi, 1984; Tanford, 1984; Inesi and de Meis, 1985).

Two approaches have been explored for the measurement of ATPase–ATPase interactions by fluorescence techniques:

(*a*)Förster type energy transfer can be demonstrated between ATPase molecules separately labeled by *N*-iodoacetyl-*N'*(5-sulfo-1-naphthyl)-ethylenediamine (IAEDANS) or iodoacetamidofluorescein (IAF) followed by reconstitution in artificial phospholipid bilayers (Vanderkooi et al., 1977). Similar observations were made by Gingold et al. (1981), Champeil et al. (1982), Watanabe and Inesi (1982), and Yantorno et al. (1983) using different donor–acceptor combinations. These observations clearly indicate ATPase interactions in reconstituted ATPase vesicles, but the structural changes induced by detergents in the membrane (Herbette et al., 1983) limit the application of the method to special circumstances.

(*b*) Excimer fluorescence of *N*-(1-pyrene)maleimide covalently bound to the Ca^{2+}-ATPase in native membranes was also suggested as probe of ATPase–ATPase interactions (Lüdi and Hasselbach, 1982, 1983). However, our studies indicate that *N*-(1-pyrene)maleimide reacts nearly randomly with several SH groups on the Ca^{2+}-ATPase; therefore, the observed excimer fluorescence may reflect intramolecular phenomena rather than ATPase–ATPase interactions (Papp et al., 1986).

The purpose of the studies reported here was to devise an energy transfer method applicable to SR that would permit dynamic measurements of ATPase–ATPase interactions under near physiological conditions without prior exposure to detergents. We choose fluorescein-5'-isothiocyanate (FITC) as energy donor and eosin-5'-isothiocyanate (EITC) as energy acceptor based on their suitable spectral characteristics (high quantum yield, significant spectral overlap) and their close structural similarity that directs their attachment to the same site on the Ca^{2+}-ATPase. FITC selectively labels a lysine residue in the putative ATP binding domain near the primary tryptic cleavage site of the Ca^{2+}-ATPase, with inhibition of the ATPase activity (Pick and Karlish, 1980, 1982; Pick and Bassilian, 1981; Mitchinson et al., 1982). Competition between EITC and

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FITC for the binding site indicates that at low reagent concentrations EITC also binds selectively to the same site. These characteristics assure that under optimal conditions about half of the ATP molecules within each vesicle is labeled by the donor and the other half by the acceptor fluorophore, and double labeling of the same ATPase molecule by acceptor and donor fluorophores is largely avoided. Therefore the energy transfer observed in FITC-EITC–labeled SR is expected to indicate ATPase–ATPase interactions, without significant contribution by intramolecular energy transfer or by energy transfer between labeled lipids and proteins. The energy transfer is inhibited by denaturation of the Ca^{2+}-ATPase or by treatment with detergents, suggesting dissociation or rearrangement of ATPase oligomers.

The principle of covalent labeling by structurally related donor–acceptor pairs that compete for the same site should prove useful in the measurement of protein–protein interactions by energy transfer in other membrane systems as well.

MATERIALS AND METHODS

Materials

FITC, EITC, and erythrosin-5′-isothiocyanate (ErITC) were obtained from Molecular Probes, Inc., Junction City, OR. 3-(*N*-Morpholino) propane sulfonic acid (MOPS), urea, rhodamine-x-isothiocyanate (RITC), lysophosphatidylcholine (LPC; egg yolk), pepsin, trypsin (type I, bovine pancreas), soybean trypsin inhibitor, adenosine-5-triphosphate, β-NADH, lactate dehydrogenase, pyruvate kinase, EGTA, Tris, Tris-maleate, and imidazole were from Sigma Chemical Co., St. Louis, MO. Sodium dodecylsulfate (SDS) was a product of Polysciences, Inc., Warrington, PA. Acrylamide, *N,N′*-methylene-bis-acrylamide, *N,N,N′,N′*-tetramethylenediamine, Coomassie Brilliant Blue R-250, and ammonium persulfate were from Bio-Rad Laboratories, Richmond, CA. Guanidine-HCl (ultra pure) was obtained from Schwarz/Mann, Spring Valley, NY, precoated glass plates (Silicagel 60) for thin-layer chromatography from E. Merck, Darmstadt, FRG, and dimethylformamide (sequanol grade) from Pierce Chemical Co., Rockford, IL. K-Oxalate, Na-vanadate (ortho), sodium deoxycholate, and bromophenol blue were from Fisher Scientific Co., Pittsburgh, PA, A23187 and $C_{12}E_8$ (octaethyleneglycol dodecylether) from Calbiochem-Behring Corp., La Jolla, CA, $^{45}CaCl_2$ from Amersham Corp., Arlington Heights, IL, and molecular weight markers for SDS polyacrylamide gel electrophoresis (PAGE) from Pharmacia Fine Chemicals Inc., Uppsala Sweden. Ludox (colloidal silica) was a gift of Du Pont de Nemours, E. I. & Co., Inc., Newton, CT.

Methods

SR vesicles were isolated from white skeletal muscles of rabbits according to Nakamura et al. (1976) and were stored before use in a medium of 0.3 M sucrose, 10 mM Tris-maleate, pH 7.0 at a protein concentration of 25–40 mg/ml at − 70°C. For protein determination the biuret (Gornall et al., 1949) and the Lowry (Lowry et al., 1951) methods were used.

The reaction of SR vesicles with FITC and its derivatives was performed as follows. The SR vesicles were suspended in 0.3 M sucrose, 50 mM Tris-HCl, pH 8.0, 5.0 mM $MgCl_2$, 0.1 mM EGTA at a final protein concentration of 2 mg/ml. The reagents for fluorescent labeling were added from concentrated (3 mM) stock solution freshly dissolved in dimethylformamide. The reaction mixtures were incubated for 30–60 min at 25°C, followed by 10-fold dilution with 20 mM K-MOPS, pH 7.0 or other buffers, as indicated in the legends. After centrifugation at 55,000–81,000 *g* for 40 min, the sedimented vesicles were resuspended in the appropriate buffer and the protein concentration was determined.

Fluorescence measurements were performed in a subnanosecond fluorescence lifetime spectrofluorometer (SLM Instruments, Inc., Urbana, IL). The temperature was controlled using a circulating waterbath (model RM-6; Lauda Div., Brinkmann Instruments Co., Westbury, NY). Fluorescence lifetimes were measured at 6, 18, and 30 MHz, using diluted Ludox suspension as scattering reference. The emission monochromator was replaced by interference filters of 520 and 540 nm for selective measurement of FITC and EITC fluorescence, respectively. Apparent phase (τp) and modulation (τm) lifetimes were obtained and analyzed using the data collection and analysis routines supplied with the instrument. The excitation and emission spectra are uncorrected.

The energy transfer efficiency and the approximate distances between donor and acceptor fluorophores were calculated assuming random orientation using donor fluorescence lifetime data determined in the presence and absence of acceptor, based on the following equation:

$$R = R_0 \cdot \left(\frac{\tau_{D,A}}{\tau_D - \tau_{D,A}} \right)^{1/6}.$$

τ_D and $\tau_{D,A}$ are the lifetimes of the donor fluorescence in the absence and presence of acceptor, respectively, and $R_0 = 53.8$ Å (Epe et al., 1983).

The ATPase activities were measured with a coupled enzyme assay essentially as described by Dean and Tanford (1977), at 28°C. The medium contained 0.1 M KCl, 20 mM Tris-HCl, pH 7.5, 0.45 mM $CaCl_2$, 0.5 mM EGTA, 5 mM ATP, 5 mM $MgCl_2$, 0.42 mM phosphoenolpyruvate, 0.2 mM NADH, 7.5 I U/ml pyruvate kinase, 18 I U/ml lactate dehydrogenase, and 1 μM A23187. The reaction was started with the addition of microsomes (0.1 μg/ml final concentration), and the absorption changes were continuously monitored at 340 nm in a Lambda 3B spectrophotometer (Perkin-Elmer Corp. Norwalk, CT). For measurement of Ca^{2+}-insensitive ATPase activity, $CaCl_2$ was omitted from the medium. In some experiments ATP hydrolysis was followed by measuring the liberation of inorganic phosphate according to Fiske and Subbarow (1925), as described earlier (Dux et al., 1985*a*).

Ca^{2+} uptake was measured at room temperature in an assay mixture containing 0.1 M KCl, 10 mM imidazole, pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, 5 mM K-oxalate, and 0.1 mM $^{45}CaCl_2$. The reaction was started by addition of SR vesicles to final protein concentration of 5–10 μg/ml, and was terminated by Millipore filtration according to Martonosi and Feretos (1964).

Trypsin digestion of SR vesicles was followed by SDS PAGE as described earlier (Dux et al., 1985*a*). Peptic proteolysis of SR vesicles and thin-layer chromatography of peptic peptides were carried out according to Martonosi (1976) and Papp et al. (1986). Fluorescent bands on SDS gels and on thin-layer chromatography plates were photographed using a long wave mercury lamp for excitation and a Wratten-2A filter for absorption of the exciting light.

RESULTS

Structures and Spectra of Fluorescent Probes

The structures of FITC, EITC, and ErITC are similar (Fig. 1). All three reagents and their analogous iodoacetamide or maleimide derivatives have been previously used for covalent labeling of the Ca^{2+}-ATPase in SR (Pick and Karlish, 1980; Mitchinson et al., 1982: Burkli and Cherry, 1981; Speirs et al., 1983; Restall et al., 1981, 1985). The three reagents preferentially react with the Ca^{2+}-ATPase in native SR and even at high reagent concentration the

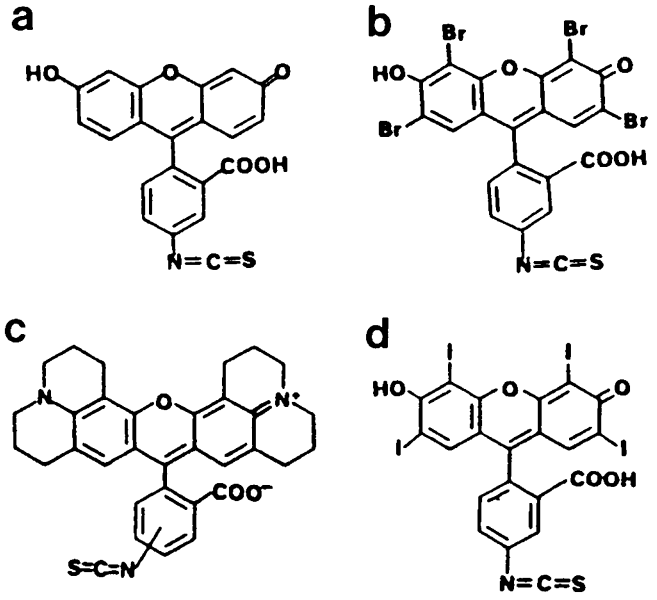

FIGURE 1 The structure of fluorescent reagents. The structure of FITC (*a*), EITC (*b*), RITC (*c*), and ErITC (*d*) are given. In the case of RITC, the proportion of isomeric forms is uncertain.

extent of covalent labeling of other proteins and phospholipids is relatively small.

The structure of rhodamine isothiocyanate (RITC) is significantly different (Fig. 1), resulting in little labeling of the Ca^{2+}-ATPase. Furthermore, due to its high lipid solubility, difficulties were encountered in the removal of unreacted RITC from the preparations. For these reasons RITC was used only in preliminary trials.

Photoexcitation of EITC or ErITC, like that of eosin or erythrosin, generates triplet states that can be quenched by oxygen, resulting in the formation of singlet oxygen (Trimer, 1985), a powerful oxidant of functional groups in the Ca^{2+}-ATPase (Yu et al., 1984; Kondo and Kasai, 1974; Restall et al., 1981; Burkli and Cherry, 1981; Kotelnikova et al., 1982; Morris et al., 1982; Watson and Haynes, 1982). The oxidation of SH and NH groups causes light-dependent inhibition of Ca^{2+} transport and ATPase activities. These effects can be prevented by exclusion of light and oxygen and addition of dithiothreitol (Restall et al., 1981; Burkli and Cherry, 1981). The photooxidation effects are most pronounced with erythrosin derivatives, while EITC and FITC are less powerful photooxidants. Therefore the experiments to be reported below were carried out with minimum exposure to light to minimize photooxidation using FITC as fluorescence donor and in most cases EITC as acceptor.

The excitation and emission spectra of the adducts of FITC, EITC, RITC, and ErITC with SR are shown in Fig. 2. The excitation and emission maxima correspond to earlier data in the literature (Burkli and Cherry, 1981; Restall et al., 1981, 1985; Speirs et al., 1983). The overlap between the emission spectrum of FITC-SR and the excitation spectrum of EITC-SR identify FITC as a suitable donor and EITC as acceptor. In some cases FITC and ErITC were also used as a donor–acceptor combination.

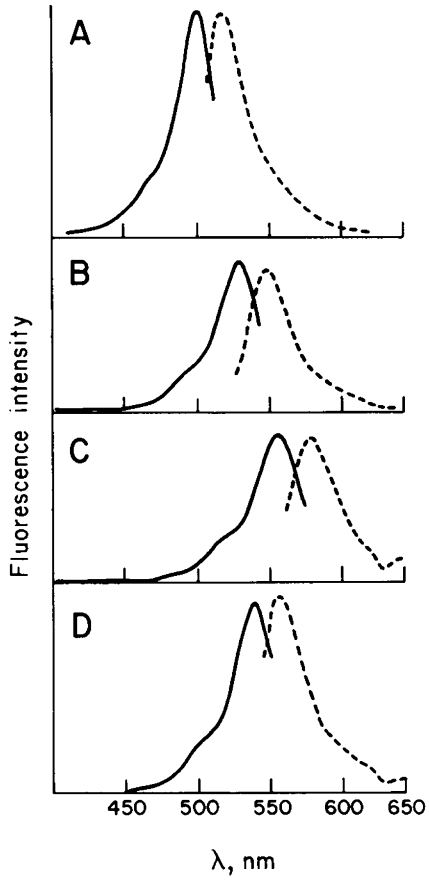

FIGURE 2 Excitation and emission spectra of SR vesicles labeled with FITC, EITC, RITC, and ErITC. SR vesicles (2 mg protein/ml) were suspended in 0.3 M sucrose, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and 0.1 mM EGTA. The fluorescent reagents were added to a final concentration of 30 nmol/mg protein from 3 mM stock solution in dimethylformamide. After incubation for 60 min at 25°C the samples were centrifuged in Spinco No. 40 rotor at 81, 000 *g* for 30 min at 2°C. The pellets were resuspended in 20 mM K-MOPS, pH 7.0 to a protein concentration of 2 mg/ml. The final dilutions to 0.05 mg/ml were done in 20 mM K-MOPS, pH 7.0 just before the fluorescence measurements at 25°C. The samples were protected from exposure to light during the procedure. The uncorrected excitation (*solid lines*) and emission (*broken lines*) spectra were taken at the following wavelengths: (*A*) FITC: λexc, 501.5 nm; λem, 517.5 nm. (*B*)EITC: λexc, 529 nm; λem, 548.5 nm. (*C*)RITC: λexc, 555.5 nm; λem, 578 nm. (*D*) ErITC: λexc, 539.5 nm; λem, 555.5 nm.

Characterization of the Adducts of FITC, EITC, ErITC, and RITC with SR

The relationship between the total concentration of FITC and EITC and the amount of fluorophore bound to the SR was determined by absorption and fluorescence measurements before and after removal of free reagents by ultracentrifugation (Table I). At total reagent concentrations of

TABLE I
THE STOICHIOMETRY OF REACTION OF FITC AND EITC WITH SR

Series	Total reagent concentration		Label bound to SR	
	First reagent	Second reagent	FITC	EITC
	nmol/mg		*nmol/mg*	
I	FITC	None		
	15		5.5	—
	30		8.4	—
	45		9.0	—
II	EITC	None		
	3.75		—	3.2
	7.50		—	6.4
	15.0		—	12.0
	30.0		—	23.6
	45.0		—	32.8
III	EITC	FITC		
	0	15	7.2	0.0
	3	15	3.1	2.0
	6	15	1.8	4.0
	12	15	1.2	8.8
	18	15	0.9	12.3
IV	FITC	EITC		
	0	15	0.0	11.1
	3	15	2.8	11.5
	6	15	4.5	10.1
	12	15	6.7	11.2
	18	15	7.7	12.9

SR vesicles were suspended in 0.3 M sucrose, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EGTA at 2–4 mg/ml protein concentration. FITC and/or EITC were added from freshly made stock solutions (3 mM in dimethylformamide) at concentrations indicated in the two left columns. Samples were incubated at 25°C for 30 min with FITC, and for 60 min with EITC. After incubation, the samples were diluted 10-fold with 20 mM K-MOPS, pH 7.0, centrifuged at 81,000 *g* for 30 min and resuspended in 20 mM K-MOPS. For calculations of the bound labels in sections I and II the absorption of supernatants were measured at 501.5 nm for FITC ($\epsilon_{501.5} = 7.0 \times 10^4\ M^{-1}\ cm^{-1}$) and at 522 nm for EITC ($\epsilon_{522} = 8.3 \times 10^4\ M^{-1}\ cm^{-1}$), and the amount of label bound to SR was calculated from the difference between the total and supernatant concentrations. For sections III and IV, the fluorescence intensities of labeled SR were measured in 20 mM K-MOPS, pH 7.0 at 25°C at a final protein concentration of 0.05 mg/ml, before and after centrifugation, using excitation wavelengths of 501.5 nm for FITC-SR and 522 nm for EITC-SR; the emission wavelengths were 517 nm for FITC-SR and 550 nm for EITC-SR. In section III the SR was labeled first with EITC, then with FITC. In section IV the order was reversed. In section I, only FITC, and in II, only EITC were used as labels.

15 nmol/mg protein for FITC or 7.5 nmol/mg for EITC, the reaction of SR with FITC or EITC is limited to 5.5 and 6.4 nmol of reagent/mg protein, respectively, corresponding to less than 1 mol reagent per mole of ATPase. Under these conditions the reaction with both reagents is confined to the Ca^{2+} transport ATPase, as shown by the distribution of fluorescence of SDS polyacrylamide gels of labeled preparations (Fig. 3). At higher reagent concentrations the amount of bound label increases beyond the level of stoichiometry with the Ca-ATPase (Table I) and on SDS

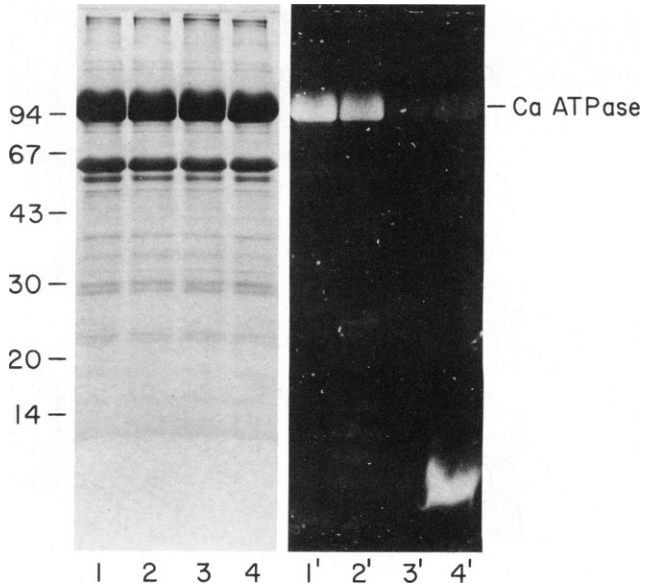

FIGURE 3. SDS polyacrylamide gel patterns of SR proteins after labeling with FITC, EITC, ErITC, and RITC. Labeling of microsomes with 15 nmol/mg of the fluorescence reagents was carried out as described in the Methods. The samples were incubated at 100°C for 5 min in SDS PAGE sample buffer without bromophenol blue, and 200-μg aliquots of protein were applied from each sample for electrophoresis. The Coomassie Blue–stained proteins (*left*) and the fluorescence patterns of the same unstained gel (*right*) are shown as follows: lanes *1* and *1'*, FITC-SR; *2* and *2'*, EITC-SR; *3* and *3'*, ErITC-SR; *4* and *4'*, RITC-SR. Numbers at left indicate molecular mass in kilodaltons.

polyacrylamide gels fluorescence is seen outside the Ca-ATPase band. Prior labeling of SR with EITC at reagent concentrations of 3–18 nmol of EITC/mg protein progressively inhibits the subsequent labeling by FITC, indicating competition between EITC and FITC for the site of attachment (Table I). After reaction of SR with 18 nmol of FITC per mg protein, EITC still binds, suggesting that when the primary reaction site of EITC is blocked, EITC continues to react at secondary sites that are not available for reaction with FITC. In the energy transfer experiments to be reported the total concentration of reagents used for labeling was usually 2.5–3.75 nmol/mg protein to minimize attachment of EITC to the secondary sites.

After incubation of SR with 15 nmol of RITC/mg protein, SDS PAGE of RITC-labeled SR reveals no association of RITC with the Ca-ATPase band (Fig. 3), and much of the RITC migrates together with phospholipids with a mobility similar to that of the free dye. It appears that much of the RITC remained unreacted, presumably associated with membrane lipids, since it could be extracted from the vesicles with chloroform-methanol (2:1, vol/vol) or with 90% acetone-10% water, and migrated as free RITC on silica gel G thin layer chromatography in two solvent systems (Fig. 4). Under identical conditions more than 90% of FITC, EITC, and ErITC was bound to membrane proteins as indicated by resistance to

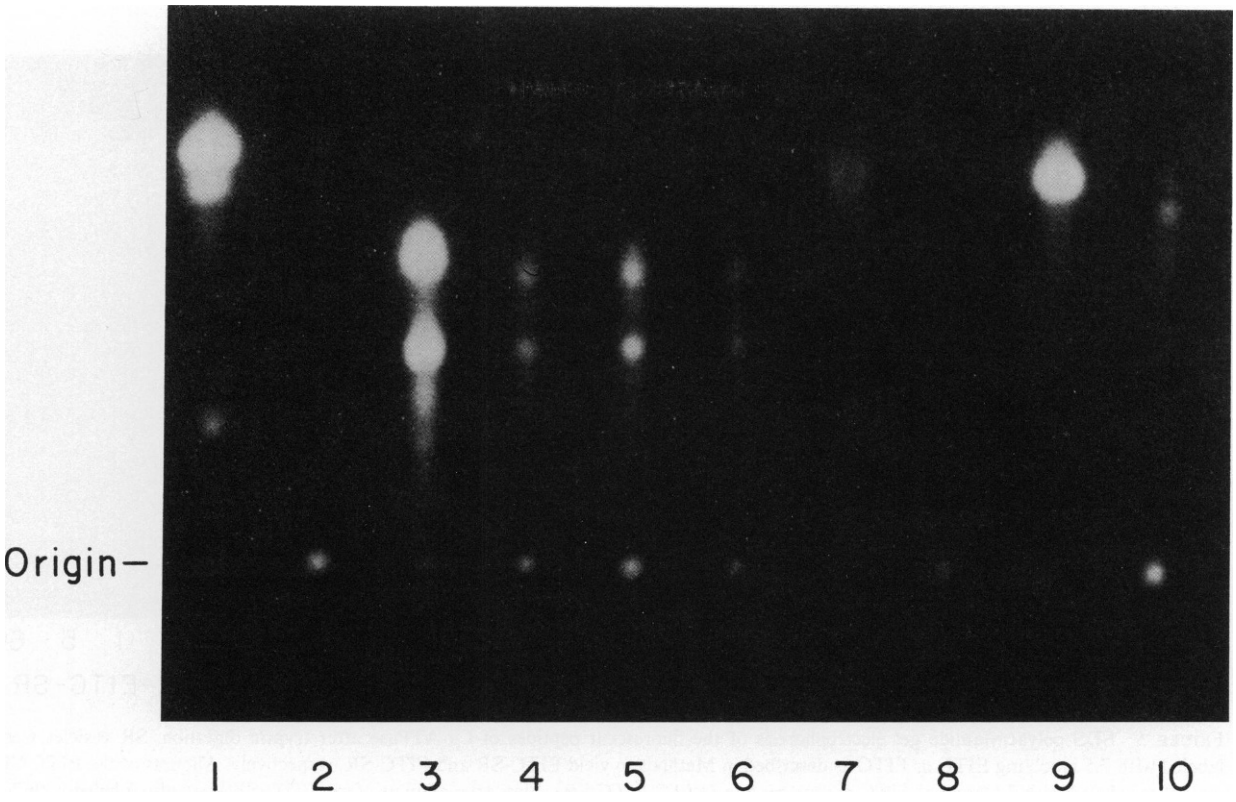

FIGURE 4 Thin-layer chromatography of the fluorescent reagents and of the labeled SR preparations. Microsomes were labeled with 30 nmol/mg of FITC, EITC, ErITC, and RITC as described under Methods. The reagents and their SR-adducts were applied on Silica gel 60 thin-layer chromatography plates and developed in a solvent system containing *n*-butanol, acetic acid, water, 5:1:2 (vol/vol). The plates were air dried and illuminated using a long wave mercury lamp for excitation. The samples are shown as follows: lane *1*, FITC; *2*, FITC-SR; *3*, RITC; *4–6*, RITC-SR after chromatography on a Sephadex G-75 column using 0.3 M sucrose, 50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$ and 0.1 mM EGTA as eluant (samples *4–6* represent the front boundary, the peak, and the rear boundary of the eluted fluorescent material, respectively); *7*, ErITC; *8*, ErITC-SR; *9*, EITC; *10*, EITC-SR. During the procedure the samples were protected against exposure to the room light. In some experiments *n*-propanol/formic acid, 80:20 (vol/vol) was used as solvent for chromatography, with similar results.

extraction with organic solvents and retention of much of the fluorescence on the line of application upon thin-layer chromatography (Fig. 4).

ErITC, at a concentration of 15 nmol/mg protein, reacted preferentially with Ca^{2+}-ATPase, but the fluorescence of the adduct was so weak (Fig. 3) that some side reaction with other proteins cannot be excluded.

Effects of Tryptic and Peptic Proteolysis

Limited tryptic proteolysis of Ca^{2+}-ATPase yields two major fragments (A and B) by cleavage at the T_1 cleavage site. FITC reacts selectively with lysine 514 in the Ca^{2+}-ATPase (MacLennan et al., 1985) at an exposed site located near the NH_2 terminus of the B fragment (Mitchinson et al., 1982). This is shown by the transfer of fluorescence after tryptic cleavage of FITC-labeled Ca-ATPase from the 110-kD region, representing the intact protein to the region of the B fragment (Fig. 5). During further digestion with trypsin the intensity of fluorescence in the B region decreases, and FITC peptides of smaller molecular size accumulate.

The distribution of fluorescence in EITC-labeled (Fig. 5) and ErITC-labeled (not shown) SR during tryptic proteolysis is very similar to that observed with FITC-SR, supporting the suggestion that FITC, EITC, and ErITC selectively reacted with the same amino acid, lysine 514, in the Ca^{2+}-ATPase.

Dual labeling of SR first with EITC and later with FITC or in reverse order, did not alter significantly the distribution of fluorescence among the peptide bands formed during tryptic hydrolysis (Fig. 5), confirming that even under these conditions both fluorophores were attached in that portion of the Ca^{2+}-ATPase that gave rise to the B fragment during tryptic proteolysis.

In peptic hydrolysates of FITC-SR and EITC-SR a tightly grouped pair of fluorescent bands was observed on thin-layer chromatography as the major fluorescent peptide products, together with trace amounts of other fluorescent peptides (not shown). Some of the side reaction may have occurred with unreacted fluorophore after the proteolysis began. Similar observations were made on tryptic hydrolysates of FITC-SR earlier by Mitchinson et al.

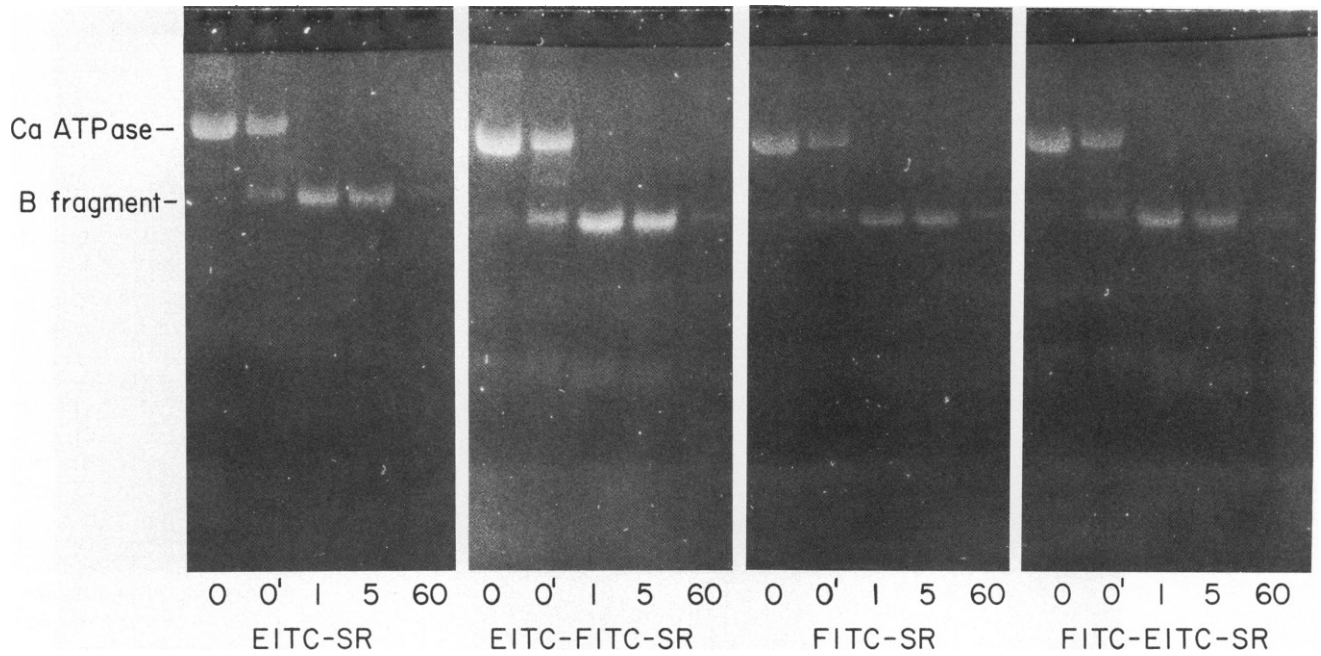

FIGURE 5 SDS polyacrylamide gel electrophoresis of the fluorescent peptides of Ca-ATPase after trypsin digestion. SR vesicles were labeled with 7.5 nmol/mg EITC or FITC as described in Methods to yield EITC-SR and FITC-SR, respectively. Aliquots of the EITC-SR were then labeled with 7.5 nmol of FITC per mg protein (*EITC-FITC-SR*). Similarly, aliquots of the FITC-SR were also labeled with 7.5 nmol EITC/mg protein (*FITC-EITC-SR*). Tryptic hydrolysis of the four preparations (4 mg protein/ml) was carried out at 25°C with 0.2 mg trypsin per ml for 0, 1, 5, and 60 min. The reaction was stopped with 0.4 mg soybean trypsin inhibitor per ml suspension. Samples marked *0* did not contain either trypsin or trypsin inhibitor. In samples marked *0'* the trypsin and trypsin inhibitor were mixed before the addition of microsomes. After digestion the samples were processed for electrophoresis and photography as described in Methods. The gel patterns of FITC-SR and EITC-SR are in essential agreement with our earlier data (Dux et al., 1985*a*), except that the less intense fluorescent bands do not show as clearly in these as in the earlier photographs.

(1982). The similarity of the peptide patterns of FITC-SR and EITC-SR is consistent with the common site of labeling by the two fluorophores.

Effects of FITC and EITC on the Ca^{2+} Transport, ATPase Activity, and Conformational Responses of SR

Lysine 514 is close to the ATP binding domain of the Ca-ATPase, and its reaction with FITC leads to inhibition of ATPase activity and Ca^{2+} transport (Pick and Bassilian, 1981; see also Table II). A similar inhibition of both functions was observed after labeling SR with EITC, while under the same conditions RITC had only slight effect (Table II).

The effect of dual labeling with FITC and EITC on the Ca^{2+}-modulated ATPase activity was tested after labeling the microsomes with the fluorophores under the three experimental conditions that were previously reported in the literature (Csermely et al., 1985; Restall et al., 1985; Burkli and Cherry, 1981). The binding of FITC and EITC to SR was nearly stoichiometric in medium I and there was a close relationship between fluorophore binding and the inhibition of ATPase activity (Table III). Therefore the reaction was performed in medium I in all experiments presented in this report. Sequential reaction with FITC followed by EITC or EITC followed by FITC produced comparable inhibition of ATPase activity to that observed when either reagent was used alone in double concentration; this further supports the suggestion that FITC and EITC interact with a common site in the Ca^{2+}-ATPase.

Although FITC and EITC apparently bind to the same

TABLE II
THE EFFECTS OF FITC, EITC, AND RITC ON Ca^{2+} TRANSPORT AND ATPASE ACTIVITY

	Ca transport	ATPase activity
	μmol Ca/mg protein	*μmol ATP/mg per min*
Control-SR	3.95	3.0
FITC-SR	0.00	0.16
EITC-SR	0.00	0.21
RITC-SR	2.59	2.81

SR vesicles (2 mg protein/ml) were labeled with 7.5 nmol of FITC, EITC, or RITC per mg protein as described in Methods. The microsomes were resuspended in 0.1 M KCl, 10 mM imidazole, pH 7.4 to a final protein concentration of 2 mg/ml and used immediately for activity measurements. Ca^{2+} uptake and ATPase activities were measured as described in Methods.

TABLE III
EFFECT OF DUAL LABELING WITH FITC AND EITC ON THE ATPASE ACTIVITY OF SR

Reagents added		Bound FITC			Bound EITC			ATPase activity		
First reagent	Second reagent	I	II	III	I	II	III	I	II	III
nmol/mg		*nmol/mg*			*nmol/mg*			*μmol Pi/mg per min*		
None	None	—	—	—	—	—	—	3.52	2.76	2.03
FITC, 2.5	EITC, —	2.6	1.1	0.8	—	—	—	1.11	1.28	1.37
FITC, 5.0	EITC, —	4.1	2.4	1.4	—	—	—	0.35	0.75	0.99
FITC, 2.5	EITC, 2.5	2.2	2.5	0.9	2.4	2.5	2.0	0.61	0.38	0.98
EITC, 2.5	FITC, —	—	—	—	2.5	2.3	2.1	1.25	1.03	1.33
EITC, 5.0	FITC, —	—	—	—	5.1	4.5	4.5	0.54	0.50	0.99
EITC, 2.5	FITC, 2.5	1.6	1.1	0.6	2.4	2.3	1.9	0.42	0.24	0.61

SR vesicles were labeled with FITC and/or EITC at a final protein concentration of 2 mg/ml in the following media: medium I, 50 mM Tris-HCl, pH 8.0, 0.3 M sucrose, 5 mM $MgCl_2$, 0.1 mM EGTA; medium II, 50 mM K-phosphate, pH 8.0; medium III, 50 mM K-phosphate, pH 8.0, 1 M KCl, 0.3 M sucrose, 5 mM $MgCl_2$, and 0.1 mM $CaCl_2$. The fluorescent reagents were added from a freshly made 3 mM stock solution in dimethylformamide and the samples were incubated at 25°C for 30 min with FITC or for 60 min with EITC. After incubation all samples were diluted 10-fold with 0.1 M KCl, 10 mM imidazole, pH 7.4, and centrifuged at 55,000 *g* for 40 min at 2°C. The pellets were resuspended in 0.1 M KCl, 10 mM imidazole, pH 7.4, and ATPase activities were measured as described under Methods. For determination of the concentration of bound reagents the SR vesicles (1 mg protein/ml) were solubilized with 5 mg deoxycholate/ml and the absorption spectra were determined using unlabeled samples as reference. The optical densities at 502 nm for FITC and 528 nm for EITC were used for the calculation of bound dye with molar extinction coefficients of $\epsilon_{502} = 7.0 \times 10^4\ M^{-1}\ cm^{-1}$ for FITC, and $\epsilon_{528} = 8.3 \times 10^4\ M^{-1}\ cm^{-1}$ for EITC. In doubly labeled samples corrections were made for the spectral overlap between the two dyes. During the procedure special care was exercised to avoid photooxidation. All buffers were degassed and bubbled with N_2, and the reactions were carried out in the dark using a Kodak 1A safety light for illumination.

site on the Ca^{2+}-ATPase, the response of the two probes to conformational changes in the Ca^{2+}-ATPase is different.

The pH and temperature dependence of the effect of Na_3VO_4 and Ca^{2+} on the fluorescence of FITC covalently bound to Ca-ATPase is consistent with the two major conformations of the Ca-ATPase predicted by kinetic studies (Pick and Karlish, 1982; Andersen et al., 1982). The E_2 conformation corresponds to the state of high fluorescence, and it is favored by EGTA + Na_3VO_4, low temperature, and high pH (Table IV). The E_1 conformation of low fluorescence is stabilized by Ca^{2+} and high temperature (Table IV).

The small decrease of fluorescence in EITC-SR caused by Ca is essentially independent of temperature, and it is of doubtful significance (Table V). EITC-SR responds to the addition of 0.2 mM Na_3VO_4 with a slight decrease in

TABLE IV
THE EFFECTS OF VANADATE AND Ca^{2+} ON FITC FLUORESCENCE AT VARIOUS TEMPERATURES AND pHs

Temperature	pH	Δ Fluorescence % of control	
		0.2 mM Na_3VO_4	0.4 mM $CaCl_2$
°C			
10	7	+11.3	−2.9
20	7	+10.4	−3.5
30	6	+ 3.1	−5.0
30	7	+ 5.0	−3.8
30	8	+ 7.2	−3.6
40	7	+ 1.0	−8.9

SR vesicles were labeled with FITC (7.5 nmol/mg protein) at 2 mg/ml protein concentration, as described in Methods. The relative fluorescence intensity of FITC was measured at a protein concentration of 50 μg/ml in 0.1 M KCl, 50–100 mM Tris-maleate, pH 6.0–8.0, 5 mM $MgCl_2$, and 0.2 mM EGTA at an excitation wavelength of 495 nm, and at an emission wavelength of 517 nm. Temperature ranged between 10° and 40°C. The changes in fluorescence intensity caused by 0.2 mM Na_3VO_4 or 0.4 mM $CaCl_2$ are expressed as percentage of control samples without addition. The free $[Ca^{2+}]$ in control samples is $\approx 10^{-8}$ M.

TABLE V
THE EFFECTS OF Na_3VO_4 AND $CaCl_2$ ON THE FLUORESCENCE OF EITC-SR AND EOSIN-LABELED SR

Temperature	pH	Δ Fluorescence % of control			
		EITC-SR		Eosin-SR	
		0.2 mM Na_3VO_4	0.4 mM $CaCl_2$	0.2 mM Na_3VO_4	0.4 mM $CaCl_2$
°C					
10	7	−1.77	−1.75	−3.2	0.0
20	7	−0.42	−1.35	−2.0	0.0
25	7	+2.40	−0.65	−4.0	0.0
30	6	+6.00	−1.05	—	—
30	7	+5.25	−0.50	−3.0	+1.0
30	8	+6.65	0.00	—	—
35	7	+6.90	−1.50	−2.0	+0.5
40	7	+6.96	−0.89	−2.0	0.0

Microsomes were labeled with EITC or with eosin at a concentration of 7.5 nmol/mg protein as described in Methods. After centrifugation the pellets were resuspended to a final protein concentration of 2 mg/ml in 0.1 M KCl, 50–100 μM Tris-maleate, pH 6–8, 5 mM $MgCl_2$, and 0.2 mM EGTA. The fluorescence measurements were carried out in the same medium at a protein concentration of 0.1 mg/ml, using an excitation wavelength of 525 nm and an emission wavelength of 550 nm for EITC and 520 and 540 nm for eosin, respectively.

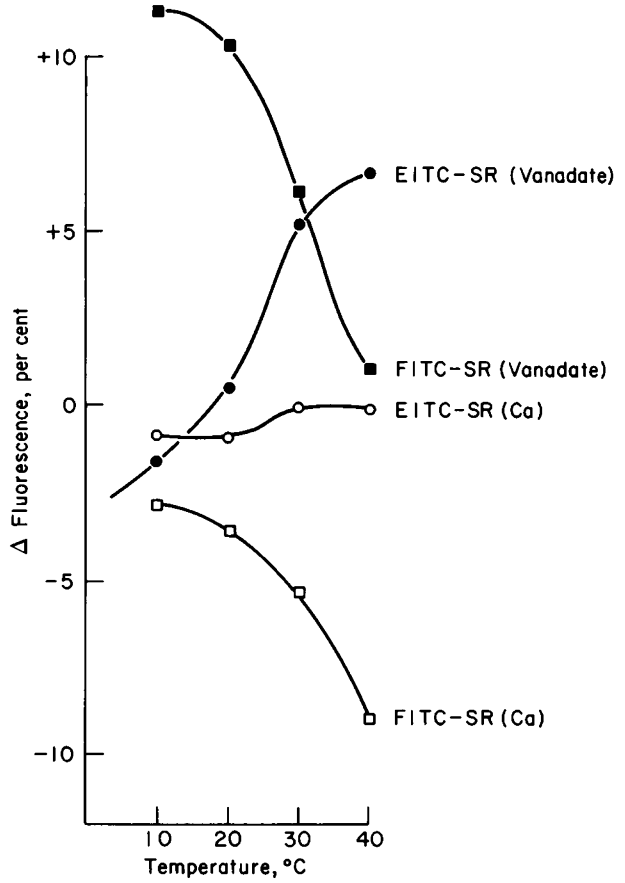

FIGURE 6 The effects of Na_3VO_4 and $CaCl_2$ on the fluorescence of FITC-SR and EITC-SR. Microsomes were labeled with 7.5 nmol/mg FITC or EITC as described in Methods, and were resuspended in 0.1 M KCl, 100 mM Tris-maleate, pH 7.0, 5 mM $MgCl_2$, and 0.2 mM EGTA, at a final protein concentration of 0.1 mg/ml. The fluorescence measurements were carried out using excitation wavelengths of 495 nm and 525 nm, and emission wavelengths of 517 nm and 550 nm for FITC and EITC, respectively. The changes in fluorescence intensities caused by 0.2 mM Na_3VO_4 or 0.4 mM $CaCl_2$ are expressed as percent of the fluorescence of control samples without addition. The effect of dilution was <0.5% in all experiments.

fluorescence intensity at 10°C and an increase in fluorescence at 30–40°C (Table V). The small changes in fluorescence of eosin-labeled SR upon addition of vanadate were independent of temperature or pH; Ca had no effect on the fluorescence of eosin-SR (Table V).

The striking differences in the temperature dependence of the fluorescences changes of FITC-SR and EITC-SR in the presence of 0.2 mM EGTA, 0.2 mM vanadate, or 0.4 mM $CaCl_2$ are illustrated in Fig. 6.

Comparison of the Excitation and Emission Spectra of FITC-SR, EITC-SR, FITC-EITC-SR, and EITC-FITC-SR

SR vesicles were labeled with FITC (FITC-SR) or EITC (EITC-SR), or with both fluorophores in different sequence (FITC-EITC-SR or EITC-FITC-SR), and their emission and excitation spectra compared (Fig. 7). The excitation maxima for FITC and EITC were at 501 and 530 nm, respectively, both in singly and doubly labeled preparations. In doubly labeled preparations excitation at 495 nm produced primarily FITC emission with secondary emission by EITC due in part to energy transfer; excitation at 524 nm produced only EITC fluorescence.

The effect of acceptor (EITC) concentration on the intensity of donor (FITC) fluorescence was measured by varying the total concentration of EITC between 0 and 7.5 nmol/mg protein at a constant FITC concentration of 2.5 nmol/mg protein (Fig. 8). With increase in EITC concentration there was a decrease in donor fluorescence intensity at 518 nm, using light of 495 nm for excitation (Figs. 8 and

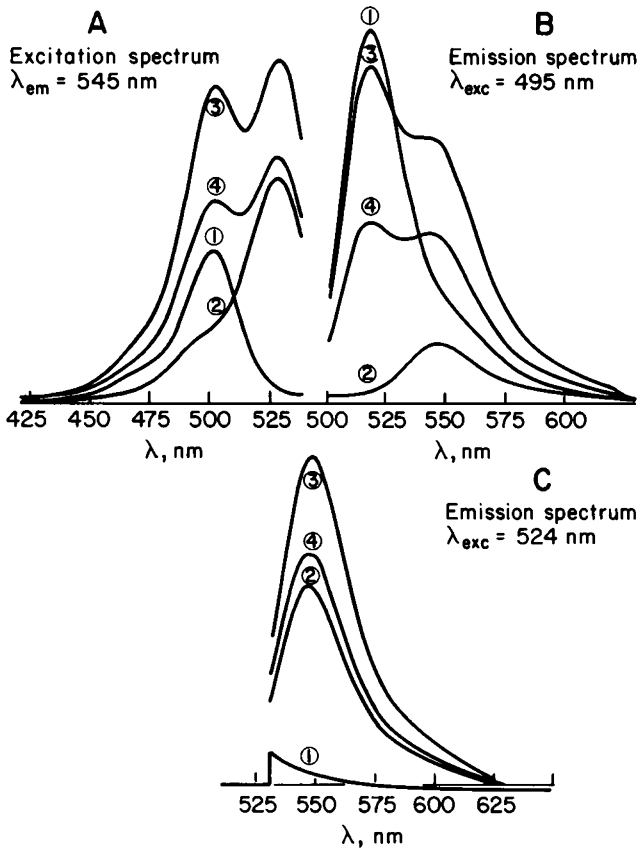

FIGURE 7 Excitation and emission spectra of SR vesicles labeled with FITC and EITC in various combinations. SR vesicles (2 mg protein/ml) in 0.3 M sucrose, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ and 0.1 mM EGTA were labeled with 3.75 nmol of FITC per mg protein for 30 min at 25°C (samples *1* and *3*). Sample *3* was subsequently labeled with EITC (3.75 nmol/mg protein) for 60 min at 25°C. Samples *2* and *4* were labeled with 3.75 nmol of EITC/mg protein for 60 min at 25°C; sample *4* was subsequently also labeled with FITC (3.75 nmol/mg protein) for 30 min at 25°C. After labeling all samples were diluted 10-fold in 20 mM K-MOPS, pH 7.0 and centrifuged at 80,000 *g* for 30 min. The pellets were resuspended in 20 mM K-MOPS, pH 7.0 to 2 mg/ml; final dilutions to 0.05 mg/ml were made with 20 mM K-MOPS, pH 7.0 just before measurements.

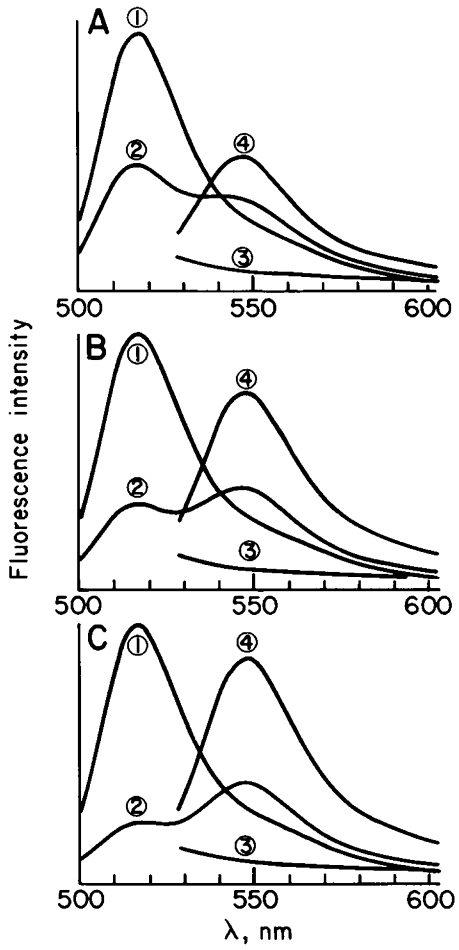

FIGURE 8 Donor and acceptor fluorescence intensities in SR vesicles labeled with FITC and EITC. SR vesicles (2 mg protein/ml) were labeled with FITC (2.5 nmol/mg protein) in a medium of 0.3 M sucrose, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and 0.1 mM EGTA at 25°C for 30 min. Aliquots of this preparation were then incubated with EITC at 2.5 (*A*), 5.0 (*B*), and 7.5 (*C*) nmol per mg protein concentration for 60 min to obtain doubly labeled preparations. After centrifugation at 81,000 *g* for 30 min the microsomes were suspended in 0.1 M KCl, 100 mM Tris-maleate, pH 7.0, 5 mM $MgCl_2$, and 0.2 mM EGTA for fluorescence measurements. In *A–C*, lines *1* and *3* represent the fluorescence emission spectra of FITC-SR with 495 and 525 nm excitation, respectively. Lines *2* and *4* are the spectra of FITC-EITC-SR preparations obtained at excitation wavelengths of 495 and 525 nm, respectively.

9). This was accompanied by a smaller increase in EITC emission at 545 nm. The change in EITC emission fluorescence approached saturation at 5 nmol of EITC per mg protein (Fig. 9). These observations are consistent with resonance energy transfer between FITC serving as energy donor and EITC as energy acceptor, but they do not give an exact measure of the efficiency of energy transfer or the distances between donor and acceptor fluorophores. Further information on the conditions of energy transfer was sought by measurement of the lifetime of donor and acceptor fluoroscence in singly and doubly labeled SR preparations.

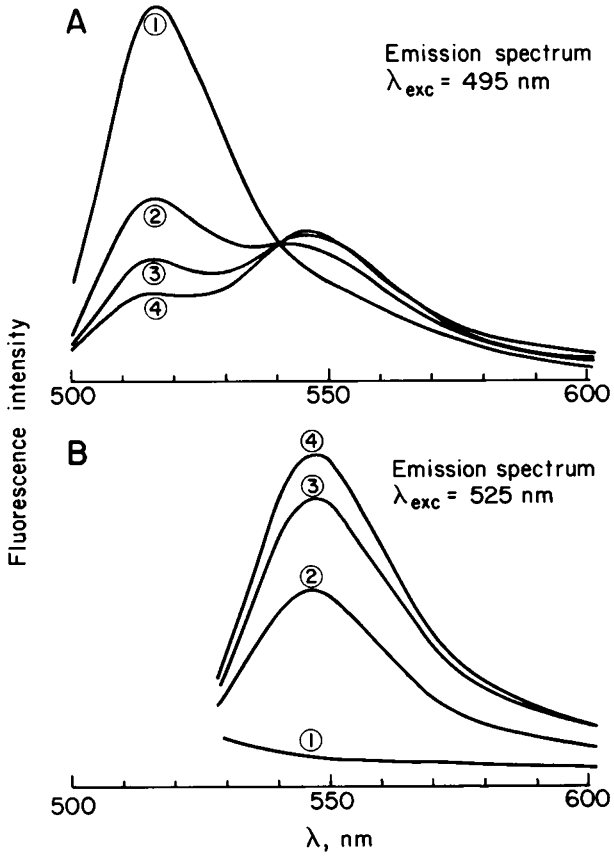

FIGURE 9 Emission spectra of SR vesicles labeled with FITC and EITC at different ratios. SR vesicles were labeled with FITC (2 nmol/mg protein) as described in Methods for 30 min at 25°C (*1*). Aliquots of this preparation were then labeled with EITC at reagent concentrations of 2.5 nmol per mg protein (*2*), 5.0 nmol per mg (*3*), and 7.5 nmol per mg (*4*) for 60 min followed by centrifugation (81,000 *g* for 30 min) and resuspension in 0.1 M KCl, 0.1 M Tris-maleate, pH 7.0, 5 mM $MgCl_2$, and 0.2 mM EGTA. Fluorescence emission spectra were recorded using light of 495 nm (*A*) or 525 nm (*B*) for excitation.

The Lifetime of Donor and Acceptor Fluorescence

The apparent phase and modulation lifetimes of FITC fluorescence were measured at 6, 18, and 30 MHz frequencies using light of 495 nm for excitation and 520 nm for emission (Table VI). Irrespective of the order of labeling the presence of EITC significantly reduced the lifetime of FITC fluorescence, consistent with efficient energy transfer between the FITC-labeled and the EITC-labeled ATPase molecules (Table VI). Based on the R_0 value of 53.8 Å for the fluorescein/eosin donor/acceptor pair (Epe et al., 1983), the observed energy transfer after labeling with 2.5 nmol/mg FITC and EITC would correspond to an average distance of 60 Å between the bond donor and acceptor fluorophores. For calculation the lifetime data of donor fluorescence, measured in the presence or absence of acceptor, were used assuming random orientation. As expected, the lifetime of EITC fluorescence (λexc, 524 nm;

TABLE VI
THE LIFETIME OF FITC FLUORESCENCE IN SR VESICLES

	Probe concentration	6 MHz		18 MHz		30 MHz	
		τp	τm	τp	τm	τp	τm
FITC-SR	2.50	3.0 ± 0.2	3.8 ± 1.1	3.0 ± 0.2	3.2 ± 0.5	3.4 ± 0.4	3.1 ± 0.9
	3.75	3.4 ± 0.1	3.6 ± 0.9	3.1 ± 0.1	3.2 ± 0.2	3.2 ± 0.2	3.5 ± 0.1
FITC EITC-SR	2.50	2.0 ± 0.3	3.6 ± 1.0	2.0 ± 0.3	2.4 ± 0.5	2.1 ± 0.3	2.4 ± 0.5
	3.75	2.0 ± 0.2	3.5 ± 0.8	2.0 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	2.6 ± 0.1
EITC-FITC-SR	2.50	2.0 ± 0.2	3.7 ± 1.0	1.9 ± 0.2	2.5 ± 0.5	2.2 ± 0.1	2.1 ± 0.4
	3.75	2.9 ± 0.3	3.3 ± 0.8	1.9 ± 0.1	2.9 ± 0.3	2.2 ± 0.0	2.9 ± 0.1

SR vesicles were labeled with FITC (FITC-SR), FITC and EITC (FITC-EITC-SR), or EITC and FITC (EITC-FITC-SR) as described in legend to Fig. 7 at total probe concentrations of 2.5 or 3.75 nmol per mg protein, respectively. Fluorescence lifetimes were measured at a protein concentration of 0.1 mg/ml in 20 mM K-MOPS, pH 7.0 at an excitation wavelength of 495 nm, using Ludox light scattering standard (IBD 1019-1069, 30–37% SiO_2, average particle diameter 15.4 nm) as scattering reference. The emitted light was isolated by P10-520-S-1383-A304 interference filter (Corion Corp., Holliston, MA) with mid-transmission at 520 nm. The apparent phase (τp) and modulation (τm) lifetimes (ns) were measured in an SLM 4800 subnanosecond fluorescence lifetime spectrophotometer at 6, 18, and 30 MHz.

λem, 540 nm) measured at 18 MHz frequency was not affected significantly by FITC (Table VII). There is reasonable agreement between τp and τm in FITC-SR (Table VI) and in EITC-SR (Table VII).

The effect of EITC on the lifetime of FITC fluorescence becomes more pronounced by increasing the EITC concentration from 2.5 nmol/mg protein to 5.0 nmol/mg protein, with slight additional increase at 7.5 nmol of EITC/mg protein (Table VIII). These observations are in accordance with the effect of EITC concentration on the intensity of donor fluorescence described in Fig. 9.

Inhibition of ATPase Interactions by Detergents, Urea, and Guanidine

The detergent $C_{12}E_8$ at a concentration of 2 mg/mg protein had no significant effect on the lifetime of FITC fluorescence in the absence of EITC, but strongly interfered with the energy transfer between EITC and FITC in doubly labeled SR preparations (Table VIII). These data indicate that $C_{12}E_8$, at the relatively high concentration used in these experiments, causes the dissociation of ATPase oligomers into monomers, in agreement with earlier ultracentrifuge and gel exclusion chromatography studies (Tanford, 1984; Martin et al., 1984; Andersen and Vilsen, 1985; Silva and Verjovski-Almeida, 1985). Vanadate had no significant effect on the lifetime of FITC fluorescence with or without $C_{12}E_8$ in the presence or absence of EITC, suggesting that the vanadate-induced shift in the E_1-E_2 equilibrium in favor of the E_2 conformation occurs without major change in the interaction between ATPase molecules.

TABLE VII
THE LIFETIME OF EITC FLUORESCENCE IN SR

	Apparent lifetimes	
	τp	τm
	ns	
EITC-SR	1.69 ± 0.15	1.65 ± 0.52
EITC-FITC-SR	1.64 ± 0.18	1.66 ± 0.40
FITC-EITC-SR	2.00 ± 0.18	1.76 ± 0.50

The labeling of microsomes with EITC and FITC was carried out as described in the legend to Fig. 7 at 3.75 nmol/mg protein total reagent concentration. The apparent phase (τp) and modulation (τm) lifetimes were measured at 18 MHz at an excitation wavelength of 524 nm using an interference filter (P10-540-S-1483-A338, Corion Corp.) to isolate the emission band centered at 540 nm.

The data of Table VIII were obtained at 10°C. Since temperature has a major effect on the conformation of Ca-ATPase (Pick and Karlish, 1982), a similar series of experiments were also performed at 20°C with similar results (Table IX). The striking inhibition by $C_{12}E_8$ of the energy transfer between FITC-labeled and EITC-labeled ATPase molecules in EITC-FITC–labeled SR preparations is also apparent in the fluorescence emission spectra obtained with light of 495 nm used for excitation (Fig. 10).

The dependence of the effect of lysophosphatidylcholine (LPC) on the energy transfer is more complex (Fig. 11). Significant inhibition of the energy transfer was observed at nonsolubilizing concentration of LPC (0.1–0.2 mg/mg protein) followed by an apparent reversal at higher LPC concentrations. There is no obvious explanation of the latter behavior.

Denaturation of the Ca^{2+}-ATPase by 8 M urea or 4 M guanidine completely abolished the energy transfer (Fig. 12).

Salyrgan at concentrations ranging between 5 and 25 nmol/mg SR protein has no significant effect on the lifetime of FITC fluorescence in FITC-SR or in EITC-FITC-SR. A slight decrease of τp was observed at higher salyrgan concentrations (50–100 nmol/mg protein), without significant change in τm, in either type of preparations. These observations imply that the inhibition of ATPase activity caused by the reaction of SH groups with salyrgan

TABLE VIII
LIFETIMES OF FITC FLUORESCENCE AT DIFFERENT CONCENTRATIONS OF EITC

Preparation	FITC concentration	EITC concentration	No addition			$C_{12}E_8$ (2 mg/mg protein)			Na_3VO_4 (200 μM)			Na_3VO_4 (200 μM) + $C_{12}E_8$ (2 mg/mg protein)		
			τp	τm	N	τp	τm	N	τp	τm	N	τp	τm	N
	nmol/mg	*nmol/mg*	*ns*	*ns*		*ns*	*ns*		*ns*	*ns*		*ns*	*ns*	
FITC-SR	2.5	—	3.51 ±0.12	3.71 ±0.26	54	3.64 ±0.12	3.85 ±0.25	18	3.62 ±0.1	3.69 ±0.22	18	3.61 ±0.13	3.82 ±0.26	36
FITC-EITC-SR	2.5	2.5	2.34 ±0.15	2.75 ±0.34	63	3.12 ±0.21	3.54 ±0.28	18	2.38 ±0.15	2.88 ±0.68	27	3.08 ±0.12	3.59 ±0.25	27
FITC-EITC-SR	2.5	5.0	1.75 ±0.15	2.74 ±0.54	32	2.82 ±0.15	3.51 ±0.46	36	1.85 ±0.12	2.15 ±0.61	5	2.62 ±0.05	3.33 ±0.14	5
FITC-EITC-SR	2.5	7.5	1.47 ±0.13	2.52 ±0.68	21	2.40 ±0.16	3.02 ±0.46	27	1.51 ±0.22	2.61 ±0.67	5	2.35 ±0.09	3.21 ±0.26	5

SR vesicles were labeled with FITC (2.5 nmol/mg protein) as described in Methods. Aliquots of this preparation were subsequently labeled with EITC at concentrations of 2.5, 5.0, and 7.5 nmol/mg for 60 min at 25°C, followed by 10-fold dilution in solution I (0.1 M KCl, 0.5 mM Tris-maleate, pH 7.0, 5 mM $MgCl_2$, 0.2 mM EGTA), and centrifugation at 80,000 *g* for 30 min. Fluorescence measurements were made in solution I at protein concentration of 0.1 mg/ml using an excitation wavelength of 495 nm and an interference filter (P10-520-S-1383-A304, Corion Corp.) to isolate the emission band centered at 520 nm. The apparent phase (τp) and modulation (τm) lifetimes were determined without further addition or after the addition of $C_{12}E_8$ (2 mg/mg protein), Na_3VO_4 (0.2 mM), or a combination of $C_{12}E_8$ and Na_3VO_4 at 18 MHz at 10°C, using Ludox suspension for scattering reference. Similar results were obtained at 20°C (not shown). *N* is the number of assays.

was not accompanied by detectable changes in the oligomeric state of the Ca^{2+}-ATPase.

Effect of Membrane Potential

Dilution of SR vesicles equilibrated in medium containing 0.15 M K-glutamate (KG) into a medium of 0.15 M choline chloride (CC) generates inside negative membrane potential; positive potential develops on dilution of microsomes from choline chloride into K-glutamate medium (Beeler et al., 1981). Membrane potential affects the ATPase and Ca^{2+} transport activity of SR (Beeler, 1980), and causes structural changes in the membrane as indicated by the effect of membrane potential on the membrane crystals of Ca^{2+}-ATPase (Dux and Martonosi,

TABLE IX
THE EFFECTS OF Na_3VO_4, Ca^{2+}, $C_{12}E_8$, AND LYSOLECITHIN ON THE LIFETIME OF FITC FLUORESCENCE

Preparation	Reagent concentration		Frequency	No addition		Na_3VO_4 (0.2 mM)		$CaCl_2$ (400 μM)		$C_{12}E_8$ (2 mg/mg protein)		Lysolecithin (1 mg/mg protein)	
	FITC	EITC		τp	τm	τp	τm	τp	τm	τp	τm	τp	τm
	nmol/mg		*MHz*	*ns*		*ns*		*ns*		*ns*		*ns*	
FITC-SR	2.5	—	18	3.30 ±0.04	3.40 ±0.15	3.51 ±0.1	3.56 ±0.22	3.34 ±0.10	3.52 ±0.12	3.51 ±0.09	3.89 ±0.15	3.54 ±0.05	4.01 ±0.10
			30	3.29 ±0.06	3.66 ±0.28	—	—	3.33 ±0.06	3.70 ±0.16	3.42 ±0.05	3.83 ±0.21	3.43 ±0.12	3.84 ±0.06
FITC-EITC-SR	2.5	2.5	18	1.73 ±0.06	1.96 ±0.22	2.27 ±0.17	2.8 ±0.31	1.82 ±0.08	2.12 ±0.41	2.84 ±0.08	3.23 ±0.17	3.02 ±0.06	3.44 ±0.12
			30	1.72 ±0.08	2.57 ±0.30	—	—	1.78 ±0.09	2.54 ±0.36	2.73 ±0.10	3.02 ±0.11	2.76 ±0.04	3.06 ±0.31
FITC-EITC-SR	2.5	5.0	18	1.45 ±0.12	2.22 ±0.36	1.77 ±0.09	2.25 ±0.5	1.47 ±0.16	2.26 ±0.52	2.58 ±0.19	3.16 ±0.53	2.55 ±0.13	3.15 ±0.38
			30	1.31 ±0.10	2.02 ±0.21	—	—	1.27 ±0.13	2.17 ±0.20	2.51 ±0.15	2.65 ±0.13	2.61 ±0.12	2.88 ±0.19
FITC-EITC-SR	2.5	7.5	18	1.36 ±0.09	2.65 ±0.65	1.34 ±0.19	2.37 ±0.5	1.61 ±0.21	2.53 ±0.35	2.33 ±0.09	2.99 ±0.36	2.26 ±0.11	3.24 ±0.30
			30	1.34 ±0.09	2.17 ±0.15	—	—	1.41 ±0.10	2.24 ±0.28	2.26 ±0.13	2.82 ±0.25	2.21 ±0.11	2.54 ±0.06

The labeling of microsomes and the measurement of apparent lifetimes was carried out as described in legend to Table VIII, except that the temperature during lifetime measurements was 20°C. For other details, see text.

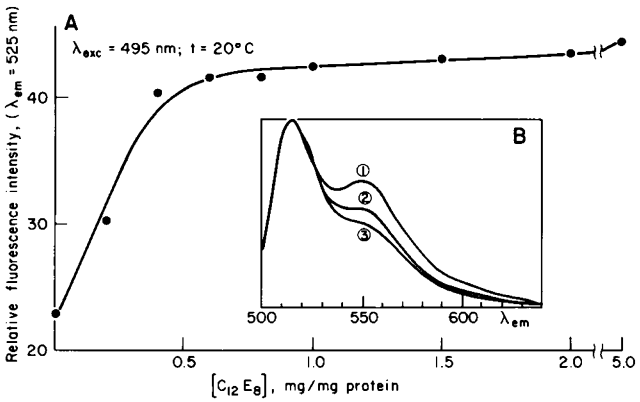

FIGURE 10 The effect of $C_{12}E_8$ on resonance energy transfer in FITC-EITC–labeled microsomes. The fluorescence emission spectra of FITC-EITC–labeled microsomes were recorded at varying $C_{12}E_8$ concentration using light of 495 nm for excitation. The relationship between $C_{12}E_8$ concentration and the fluorescence intensity of FITC at 515 nm is shown in *A*. In the insert (panel *B*) emission spectra are presented normalized to the 515 nm peak. The spectra were recorded in the absence of $C_{12}E_8$ (*1*) and in the presence of $C_{12}E_8$ at concentrations of 0.2 mg/mg protein (*2*) and 0.4 mg/mg protein or higher (*3*). Temperature, 20°C.

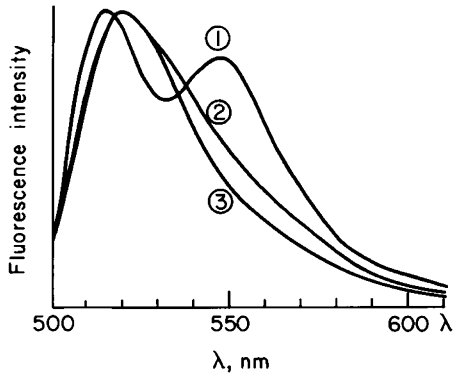

FIGURE 12 The effect of urea and guanidine on resonance energy transfer in FITC-EITC–labeled SR. SR vesicles were labeled first with FITC and then with EITC, both at a concentration of 2.5 nmol/mg protein, as described in Methods. The emission spectra were measured in a medium of 0.1 M KCl, 0.1 M Tris-maleate, pH 7.0, 5 mM $MgCl_2$, 0.2 mM EGTA at a protein concentration of 0.05 mg/ml, using light of 495 nm for excitation. The spectra were normalized with respect to 515 nm. *1*, Control sample, no addition. *2*, Same with 8 M urea. *3*, Same with 4 M guanidine.

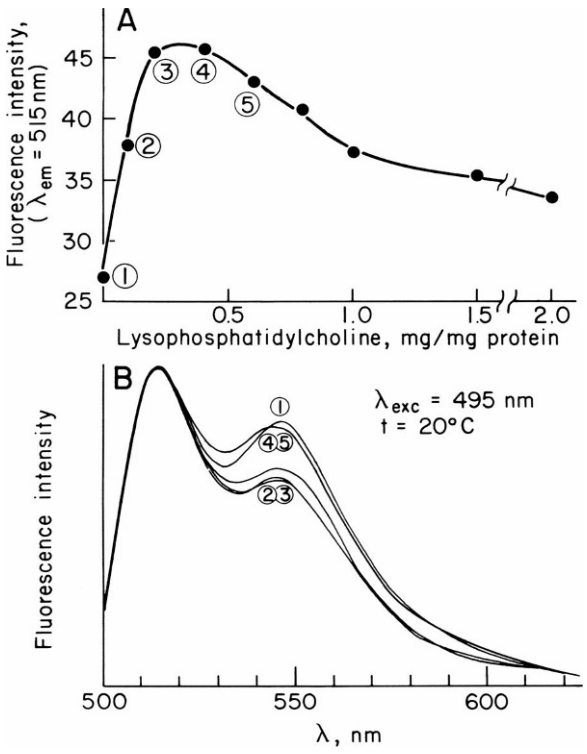

FIGURE 11 The effect of LPC on the energy transfer in FITC-EITC–labeled microsomes. FITC-EITC–labeled microsomes were prepared and the fluorescence emission spectra were recorded as described in legend to Fig. 8 using light of 495 nm for excitation. Temperature, 20°C. (*A*) The fluorescence intensity at 515 nm sharply increased with LPC concentration up to 0.2 mg/mg protein, reaching a plateau, followed by a decrease in fluorescence intensity at LPC concentration greater than 0.4 mg/mg protein. (*B*) The emission spectra (normalized to 515 nm) are given for the following LPC concentrations (mg/mg protein): 0 (*1*), 0.1 (*2*), 0.2 (*3*), 0.4 (*4*), and 0.8 (*5*).

1983*d*; Dux et al., 1985*b*; Beeler et al., 1984). FITC-labeled SR responds to positive membrane potential with an increase and to negative membrane potential with a decrease in the intensity of FITC fluorescence; these changes relax over a period of 10–15 min with the dissipation of membrane potential due to ion fluxes across the membrane (Jona and Martonosi, 1986).

Based on these observations we investigated the effect of membrane potential induced by ion substitution on the interaction between ATPase molecules by measuring the energy transfer in FITC-EITC–labeled SR (Table X). The lifetimes (τp and τm) of FITC fluorescence in FITC-SR, FITC-EITC-SR, and EITC-FITC-SR were unaffected by ion substitution under conditions expected to yield zero, positive, or negative transmembrane potentials. For technical reasons lifetime measurements could not be made during the first 40 s immediately after the dilution of microsomes, when the potential changes were the greatest. Nevertheless, the absence of significant changes in the fluorescence lifetime in the period ranging between 1 and 15 min after dilution exclude major potential-dependent changes in the oligomeric state of Ca^{2+}-ATPase.

DISCUSSION

The interactions between ATPase molecules in SR are well documented, but the functional and structural significance of these interactions remains elusive, and little is known about the conditions that influence the monomer–oligomer equilibrium of Ca^{2+}-ATPase (Møller et al., 1982; Martonosi and Beeler, 1983; Martonosi, 1984; Tanford, 1984; Inesi and de Meis, 1985). The relatively slow progress in this area is largely due to the lack of satisfactory techniques that would permit accurate determination of the size distribution of ATPase oligomers, the equilibrium

TABLE X
EFFECT OF MEMBRANE POTENTIAL ON THE LIFETIME OF FITC FLUORESCENCE IN FITC-SR, FITC-EITC-SR, AND EITC-FITC-SR

Ion substitution	Time after dilution	FITC-SR		FITC-EITC-SR		EITC-FITC-SR	
		τp	τm	τp	τm	τp	τm
	min	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
KG-KG	1	3.17	3.11	2.68	2.46	2.72	2.64
	2	2.86	2.39	2.23	2.73	2.24	—
	3	3.42	3.33	2.49	1.83	2.37	2.27
	5	2.97	2.69	2.07	2.12	2.39	2.71
	10	3.13	4.00	2.08	2.91	2.13	—
	15	3.12	3.06	2.23	2.39	2.20	2.01
CC-CC	1	3.51	3.29	2.38	2.42	2.10	2.07
	2	3.39	3.53	2.25	2.65	2.43	2.62
	3	3.59	3.58	2.46	2.54	2.58	2.42
	5	3.37	3.70	2.34	2.32	2.77	2.66
	10	3.62	3.23	2.15	2.37	2.68	2.75
	15	3.48	3.43	2.42	—	2.62	3.10
KG-CC	1	3.51	3.15	2.39	3.23	2.50	2.72
	2	3.18	—	2.50	3.02	2.39	2.44
	3	3.43	4.12	2.32	2.66	2.41	2.98
	5	2.83	3.88	2.30	2.39	2.26	2.70
	10	3.41	3.68	2.32	2.20	2.54	2.75
	15	3.26	3.83	2.50	2.87	2.32	2.38
CC-KG	1	3.49	3.58	2.59	2.27	2.41	2.72
	2	3.19	3.81	2.31	3.42	2.55	2.86
	3	3.44	2.93	2.27	2.90	2.41	2.65
	5	3.40	3.72	2.15	2.82	2.36	2.79
	10	3.26	3.88	2.35	3.13	2.54	2.77
	15	3.17	4.09	2.53	2.80	2.55	3.13

SR vesicles (2 mg protein/ml) were labeled with 2.5 nmol of FITC and/or EITC per mg protein as described under Methods. The labeled samples were diluted 10-fold with one of the following media: KG medium: 150 mM K-glutamate, 10 mM K-MOPS, pH 7.0, 5 mM $MgCl_2$, and 0.5 mM EGTA. CC medium: 150 mM choline chloride, 10 mM Tris-MOPS, pH 7.0, 5 mM $MgCl_2$, and 0.5 mM EGTA. The diluted samples were centrifuged at 55,000 *g* for 40 min at 2°C. The pellets were resuspended in the dilution medium to a final protein concentration of 20 mg/ml. After 3 h at 2°C, SR vesicles dispersed in KG medium (KG microsomes) were diluted 200-fold into CC medium to generate inside negative potential; KG microsomes diluted into KG medium served as control (zero transmembrane potential). Similarly SR vesicles dispersed in CC medium (CC microsomes) were diluted into KG medium to generate inside positive membrane potential; CC microsomes diluted into CC medium served as controls (zero transmembrane potential). The apparent phase (τp) and modulation lifetimes (τm) of FITC were measured at 10°C using 18 MHz modulation frequency as described in Methods. The lifetime (ns) measurements were initiated within 50 s after the generation of potential by dilution and repeated lifetime measurements were made during a 15-min period after dilution.

constant of ATPase–ATPase interactions, and the rate constants of the formation and decomposition of ATPase oligomers in the intact SR under near physiological conditions. The problem is not unique to SR, since protein–protein interactions are common features of all biological membranes.

The analysis of ATPase interactions by ultracentrifuge (Martin, 1983) or gel filtration techniques (Silva and Verjovski-Almeida, 1985; Andersen and Vilsen, 1985) requires prior solubilization of the membrane by detergents that change the structure of membrane proteins (Mommaerts, 1967; Masotti et al., 1972; Hardwicke and Green, 1974; le Maire et al., 1976; Dean and Tanford, 1978; Nakamoto and Inesi, 1986; Csermely et al., 1987) and cause disruption or rearrangement of the interactions between them. Therefore even if the ATPase activity is preserved in solubilized systems (Møller et al., 1982; Tanford, 1984), they are not likely to represent reliable models for the analysis of protein–protein interactions.

Electron microscope observations on crystalline Ca^{2+}-ATPase arrays in the intact SR yielded interesting structural information on the types of interactions that occur when the ATPase molecules are frozen in the E_1 and E_2 conformations (Dux and Martonosi, 1983, *a–d;* Dux et al., 1985*b;* Taylor et al., 1984, 1986; Castellani et al., 1985; Ferguson et al., 1985; Franzini-Armstrong and Ferguson, 1985). However, it is difficult to predict the relevance of the interactions within the conformationally uniform crystalline arrays to the ATPase–ATPase interactions that occur in a Ca^{2+}-transporting membrane, where cyclic structural changes in Ca^{2+}-ATPase generate a dynamic coexistence of ATPase molecules in different conformational states.

Spectroscopic methods may offer the combination of

sensitivity and kinetic resolution that is needed for the measurement of ATPase–ATPase interactions in functionally competent membranes under near physiological conditions.

Resonance energy transfer between pairs of fluorophores of appropriate excitation and emission characteristics proved useful for distance measurements in protein molecules and within macromolecular complexes (Stryer, 1978). In an earlier application of this method for the assessment of distances between Ca^{2+} transport ATPase molecules in reconstituted membranes (Vanderkooi et al., 1977), one portion of a Ca^{2+} transport ATPase preparation was labeled with IAEDANS as fluorescence energy donor and another portion with IAF as fluorescence energy acceptor. In reconstituted vesicles containing both donor- and acceptor-labeled ATPase molecules, fluorescence energy transfer was observed as judged by the ratio of donor and acceptor fluorescence intensities and by the effect of acceptor-labeled ATPase molecules on the lifetime of donor fluorescence (Vanderkooi et al., 1977). The energy transfer implies interactions between ATPase molecules. These observations were confirmed and extended with the use of other fluorophores in several laboratories (Gingold et al., 1981; Champeil et al., 1982; Watanabe and Inesi, 1982; Yantorno et al., 1983). The principal drawback of this technique is that the disposition of ATPase molecules in reconstituted vesicles is different from that of native membranes (Herbette et al., 1983), raising uncertainty whether the observed energy transfer reflects physiologically relevant interactions between ATPase molecules.

The studies reported here were designed to extend the resonance energy transfer methodology to native SR by the use of structurally related pairs of donor and acceptor fluorophores, that compete specifically for a single site on the Ca^{2+}-ATPase. Based on structural evidence for the selective reaction of FITC with lysine 514 (MacLennan et al., 1985; Brandl et al., 1986), we chose FITC as energy donor and the structural analogues EITC and ErITC as acceptors. The following observations indicate that FITC and EITC fulfill most of the requirements as resonance energy transfer pair for steady-state and kinetic analyses of ATPase interactions in native SR:

(*a*) At low labeling ratios (2.5–3.5 nmol/mg protein), FITC and EITC selectively and competitively label the same region of the Ca^{2+}-ATPase, with only slight side reactions that are not likely to contribute significantly to energy transfer. Since simultaneous reaction of both donor and acceptor fluorophores on the same ATPase molecule is minimized the observed energy transfer can be attributed to interactions between distinct, donor-labeled and acceptor-labeled ATPase molecules.

(*b*) The spectral characteristics of the fluorophores assure readily measurable energy transfer either by donor/acceptor fluorescence intensity ratio, or by donor fluorescence lifetime determinations.

(*c*) The energy transfer is significantly reduced by low concentration of detergents that cause dissociation of ATPase oligomers and it is completely abolished by denaturation of Ca^{2+}-ATPase with urea or guanidine.

The energy transfer was not affected significantly by EGTA + vanadate, that stabilize the E_2 conformation, or by Ca^{2+} and lanthanides that stabilize the E_1 conformation of the Ca^{2+}-ATPase. Therefore the cyclic changes in enzyme conformation are not likely to be associated with changes in the monomer-oligomer equilibrium of Ca^{2+}-ATPase.

Changes in transmembrane potential by ion substitution were also without significant effect on ATPase–ATPase interactions, although potential dependent changes in the conformation of Ca^{2+}-ATPase were observed (Jona and Martonosi, 1986).

Labeling of the Ca^{2+}-ATPase with FITC or EITC inhibits the ATPase activity and Ca^{2+} transport by blocking the ATP binding site (Pick and Karlish, 1980, 1982; Pick and Bassilian, 1981). The FITC-labeled enzyme retains the characteristic conformational responses to Ca, EGTA, and vanadate that are connected with transitions between the E_1 and E_2 states (Pick and Karlish, 1982; Jona and Martonosi, 1986). FITC-labeled Ca^{2+}-ATPase can also be induced by EGTA and vanadate to form E_2 type crystals that are indistinguishable from the E_2 crystals in native SR (Csermely et al., 1985; Varga et al., 1987). Similar observations were made on SR doubly labeled with FITC and EITC at a reagent concentration of 2.5 nmol/mg protein. Therefore it is reasonable to assume that the conformational responses and interactions of the native SR are preserved after the binding of FITC to the Ca^{2+}-ATPase. The inhibition of Ca transport is likely to result from a localized direct interference by FITC or EITC with the binding of ATP. Therefore the ATPase–ATPase interactions indicated by the energy transfer probably represent the properties of native SR.

Future studies will be directed at the identification of structurally analogous donor–acceptor pairs that react specifically with the Ca-ATPase at non-essential sites without interference with its enzymatic activity.

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